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# Effect of cell immobilization and pH on *Scheffersomyces stipitis* growth and fermentation capacity in rich and inhibitory media

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## Abstract

**Background:** A wide range of value-added products can potentially be produced by bioprocessing hardwood spent sulfite liquors (HSSLs) that are by-products of pulp and paper industry with a high pentose sugar content. However, besides sugars, HSSLs contain considerable amounts of sulfonated lignin derivatives and acetic acid that inhibit the metabolic activity of most microorganisms. *Scheffersomyces stipitis* is a yeast with high capacity to ferment the pentose sugar xylose under appropriate microaerophilic conditions but it has limited tolerance to HSSL inhibitors. In the present study, cultivations of suspended and immobilized *S. stipitis* were compared in terms of growth capacity and by-product formation using rich medium and HSSL to investigate whether the immobilization of cells in calcium alginate beads could be a protection against inhibitors while favoring the presence of microaerophilic conditions.

**Results:** Whereas cell immobilization clearly favored the fermentative metabolism in rich medium, pH control was found to play a more important role than cell immobilization on the ethanol production efficiency from bio-detoxified HSSL (*bd*HSSL), leading to an improvement of 1.3-fold on the maximum ethanol productivity than using suspended cells. When immobilization and pH control were applied simultaneously, the ethanol yield improved by 1.3-fold with unchanged productivity, reaching  $0.26 \text{ g ethanol} \cdot (\text{g glucose} + \text{xylose})^{-1}$ . Analysis of the immobilized beads inside revealed that the cells had grown in the opposite direction of the cortex.

**Conclusions:** Immobilization and pH control at 5.5, when applied simultaneously, have a positive impact on the fermentative metabolism of *S. stipitis*, improving the ethanol production efficiency. For the first time light microscopic analysis of the beads suggested that the nutrient and mass transfer limitations played a more important role in the fermentation than a possible protective role against inhibitors.

**Keywords:** *Scheffersomyces stipitis*; Hardwood spent sulfite liquor; Cell immobilization; Light microscopy; Ca alginate beads; Xylose fermentation; Stress tolerance

## Background

Spent sulfite liquors (SSLs) are by-products that result from the acidic wood cooking process containing high amounts of fermentable sugars [1,2]. SSLs are by-products with low-cost associated, being potential substrates for the production of several value-added compounds. Among them, bioethanol - a renewable, biodegradable and non-toxic biofuel - derived from SSLs

might constitute a cost-effective and large-scale production alternative, with associated environmental benefits [1,3].

The chemical composition of SSLs depends on the wood species used in the pulping process. Typically, undiluted hardwood SSLs (HSSLs) from *Eucalyptus globulus* contain 35 to 45  $\text{g.L}^{-1}$  sugars, mainly xylose [2,4]. This significant xylose content makes HSSL a suitable substrate for pentose-fermenting yeasts, such as *Scheffersomyces stipitis* [2,5]. However, up to 8.0 to 10.0  $\text{g.L}^{-1}$  acetic acid and 59.0 to 78.8  $\text{g.L}^{-1}$  lignosulfonates have also been reported and they both act as inhibitors of microbial metabolism. In fact, the wood cooking process

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uses harsh conditions (pH 1 to 2, 125°C to 135°C, 8 to 12 h), which leads to the formation of microbial inhibitory chemical compounds [2]. Notably aromatic, polyaromatic, phenolic, and aldehyde compounds can be generated from lignin and the hemicellulose fraction degradation, thus affecting the integrity of biological membranes and inhibiting yeast metabolism [6-8]. Several studies reported a synergistic effect on inhibition when the furan derivatives, weak acids and/or phenolic compounds were simultaneously present [9-12].

*S. stipitis* was described as having the highest native capacity for D-xylose fermentation of any known microbe [1,13]. This yeast induces fermentative activity in response to oxygen limitation and reaches the optimal activity with microaerophilic conditions (1% to 15% O<sub>2</sub>) [14,15]. One of the major drawbacks of *S. stipitis* is the low tolerance towards inhibitors and high ethanol concentrations [16]. *S. stipitis* performances in untreated HSSLs are strongly affected, since growth occurs at a considerable lower level and the ethanol yields can only reach 30% to 50% of the theoretical maximum - 0.51 g ethanol.g sugars<sup>-1</sup> [1,17].

Physico-chemical detoxification of HSSL, metabolic engineering and strain adaptation are possible strategies to improve the alcoholic fermentation efficiency in yeast species [4,7,18-20]. Biological detoxification of HSSL with the fungus *Paecilomyces variotii* was also found to improve the ethanol production by *S. stipitis* [4]. Finally, cell immobilization could be an advantageous approach to ferment lignocellulosic by-products with inhibitors, improving cell stability, activity and stress tolerance [21-23]. In the case of cell entrapment, the layer creates a microenvironment that acts as a protection for high ethanol concentrations and inhibitors limiting mass transfer phenomena. However, it might also affect the oxygen mass transfer. Facilitated product recovery, the possibility to reuse the biomass and the lower risk of microbial contamination are other advantages of cell immobilization that could compensate for the additional investment that this process involves [24].

The aim of the present study was to investigate the effects of cell immobilization and pH control on the performances of *S. stipitis* in rich medium and in bio-detoxified HSSL (*bdHSSL*). Immobilization was done by Ca alginate entrapment in order to investigate if it could limit the oxygen available for the cells, and therefore favor the fermentative metabolism of *S. stipitis*, and/or confer a protection layer against the inhibitory compounds that were still present in the *bdHSSL*. Growth and by-product formation using suspended and calcium alginate immobilized cultures of *S. stipitis* were compared and light microscopy was used to analyze the inside of beads in order to observe the mass transfer effects.

## Methods

### Strain

*S. stipitis* NRRL-7124 was supplied by the Agricultural Research Service Culture Collection at National Center for Agricultural Utilization Research, USDA. Stock cultures were stored in 30% glycerol at -80°C. *S. stipitis* culture was grown for 48 h at 28°C and maintained at 4°C in YM (yeast medium) agar plates containing 10 g.L<sup>-1</sup> glucose, 5 g.L<sup>-1</sup> peptone, 3 g.L<sup>-1</sup> malt extract, 3 g.L<sup>-1</sup> yeast extract and 20 g.L<sup>-1</sup> agar.

### Hardwood spent sulfite liquor

HSSL (Caima SA- ALTRI Group, Constância, Portugal) was obtained from a magnesium-based *E. globulus* wood cooking process. HSSL was physico-chemically pretreated as described by Xavier et al. in 2010 [5]. After the pretreatment, HSSL contained 24.6 g.L<sup>-1</sup> xylose, 8.5 g.L<sup>-1</sup> mannose, 7.8 g.L<sup>-1</sup> arabinose, 4.5 g.L<sup>-1</sup> galactose and 2.3 g.L<sup>-1</sup> glucose [5]. Besides these sugars, HSSL contained the following potential inhibitory compounds: 78.2 g.L<sup>-1</sup> lignosulfonates, 9.6 g.L<sup>-1</sup> acetic acid and traces of 5-hydroxymethyl-2-furaldehyde and 2-furaldehyde [5]. Therefore further HSSL bio-detoxification was performed with a filamentous fungus as reported by Pereira et al. in 2012 [4].

### Yeast cultures

YMglc and YMxyl (10 g.L<sup>-1</sup> glucose or xylose, 3.0 g.L<sup>-1</sup> malt extract and 3.0 g.L<sup>-1</sup> yeast extract) were used as pre-culture medium for the rich medium cultivations. YNBglc medium containing 20 g.L<sup>-1</sup> glucose, 6.7 g.L<sup>-1</sup> yeast nitrogen base without amino acids, 10.2 g.L<sup>-1</sup> potassium hydrogen phthalate and 2.2 g.L<sup>-1</sup> KOH was used as pre-culture medium for the *bdHSSL* cultivations. The inocula were prepared taking a single colony from the YM plate into YNBglc. Cells were grown until the culture reached the beginning of the stationary phase at 30°C and 180 rpm.

### Cultivations

#### Rich medium cultivations

Pre-cultures were performed in 100 mL of YMglc or YMxyl in 250 mL shake flasks. Cultures using free or immobilized cells were carried out in duplicate in 200 mL of YMglc or YMxyl in 250 mL shake flasks with a starting OD<sub>620nm</sub> of 0.5 to 0.6. In the case of immobilized cells, the cell wet pellet correspondent to the mentioned OD was gently mixed with 2.8% sodium alginate and after complete homogenization, the mixture was added to 2.0% CaCl<sub>2</sub> using sterile needles and a distance needle solution of 4 to 7 cm. After 20 min with gentle agitation, the beads were transferred into 0.5% CaCl<sub>2</sub> and process was repeated (20 min, gentle agitation). The beads were then sterile transferred to the cultivation

medium.  $OD_{620nm}$ , pH and metabolites concentration were followed. Samples for high performance liquid chromatography (HPLC) were centrifuged and the supernatant stored at  $-20^{\circ}C$  until further analysis.

### Screening in *bdHSSL*

After pre-cultures, *S. stipitis* was inoculated to six different *bdHSSL* levels (0%, 20%, 40%, 60%, 80%, and 100%) at an initial  $OD_{620nm}$  of 0.5. The media were prepared using YNB buffer 10 $\times$  (10.2 g.L $^{-1}$  potassium hydrogen phthalate, 2.2 g.L $^{-1}$  KOH), YNB 10 $\times$  (6.7 g.L $^{-1}$  yeast nitrogen base), *HSSL* sugars solution 5 $\times$  (123.0 g.L $^{-1}$  xylose, 42.5 g.L $^{-1}$  mannose, 39.0 g.L $^{-1}$  arabinose, 22.5 g.L $^{-1}$  galactose, 11.5 g.L $^{-1}$  glucose). Aerobic growth cultures were performed in 50 mL conical tubes containing 5 mL of media. Cells were grown at  $30^{\circ}C$  and 180 rpm and  $OD_{620nm}$  was followed over time. The pH was determined at the end of the experiments. All cultivations were performed in duplicate.

### *bdHSSL* cultivations

Pre-cultures were performed in 50 mL conical tubes or 250 mL baffled flasks and were used to inoculate 100 mL of 60% *bdHSSL* at an initial  $OD_{620nm}$  of 0.5. Cells were grown at  $28^{\circ}C$  and at the beginning of the stationary phase a cell pellet was obtained in order to inoculate undiluted *bdHSSL* with  $7.3 \times 10^{-2}$  g CWW.mL $^{-1}$  (g cell wet weight. mL $^{-1}$ ), approximately  $10^8$  CFUs. mL $^{-1}$  (colony forming units per mL), which corresponds to an amount of cells that bio-industries use to start bioprocesses. Usually, this process is done by cell dry weight measurement but in this case it would be impossible since the cells were reutilized to start the cultivation assays.

Besides the assays with suspended cells, *S. stipitis* cells were also immobilized by entrapment in calcium alginate gel beads (as described in the 'Rich medium cultivations' section). The same procedure in order to obtain the correct CWW to start the cultivation was used as for the suspended cultures. When immobilized cells were used, the *bdHSSL* was previously supplemented with 0.2% CaSO $_4$ .

Cultivations with both suspended and immobilized cells were also performed controlling the pH. KOH 3 M or H $_2$ SO $_4$  3 M were pumped by an automatic control system in order to maintain the pH at 5.5.

Batch cultivations were carried out, in duplicate, in 1,000 mL fermenters with a working volume of 400 mL, at  $30^{\circ}C$ , initial pH of 5.5, airflow of 0.3 vvm and 160 to 180 rpm.  $OD_{620nm}$  and pH were measured over time. Samples for HPLC were centrifuged and the supernatant stored at  $-20^{\circ}C$  until further analysis.

### HPLC analysis

Concentrations of glucose, xylose, ethanol, acetate, xylitol and glycerol were determined by using a Eurokat H

10  $\mu$ m 300  $\times$  8 column (Knauer, Berlin, Germany) with H $_2$ SO $_4$  0.01 N at 0.4 mL.min $^{-1}$  as mobile phase at  $40^{\circ}C$ . The injected volume was 20  $\mu$ L. HPLC (VWR-Hitachi LaChrom Elite $^{\circ}$  -Tokyo, Japan) system was equipped with a Pump L-2130 and auto-sampler L-2200 from Hitachi (Tokyo, Japan), oven Gecko 2000 (Hattersheim, Germany) set at  $40^{\circ}C$  and refraction index (RI) detector L-2490 (Hitachi-Tokyo, Japan).

### *S. stipitis* beads analysis

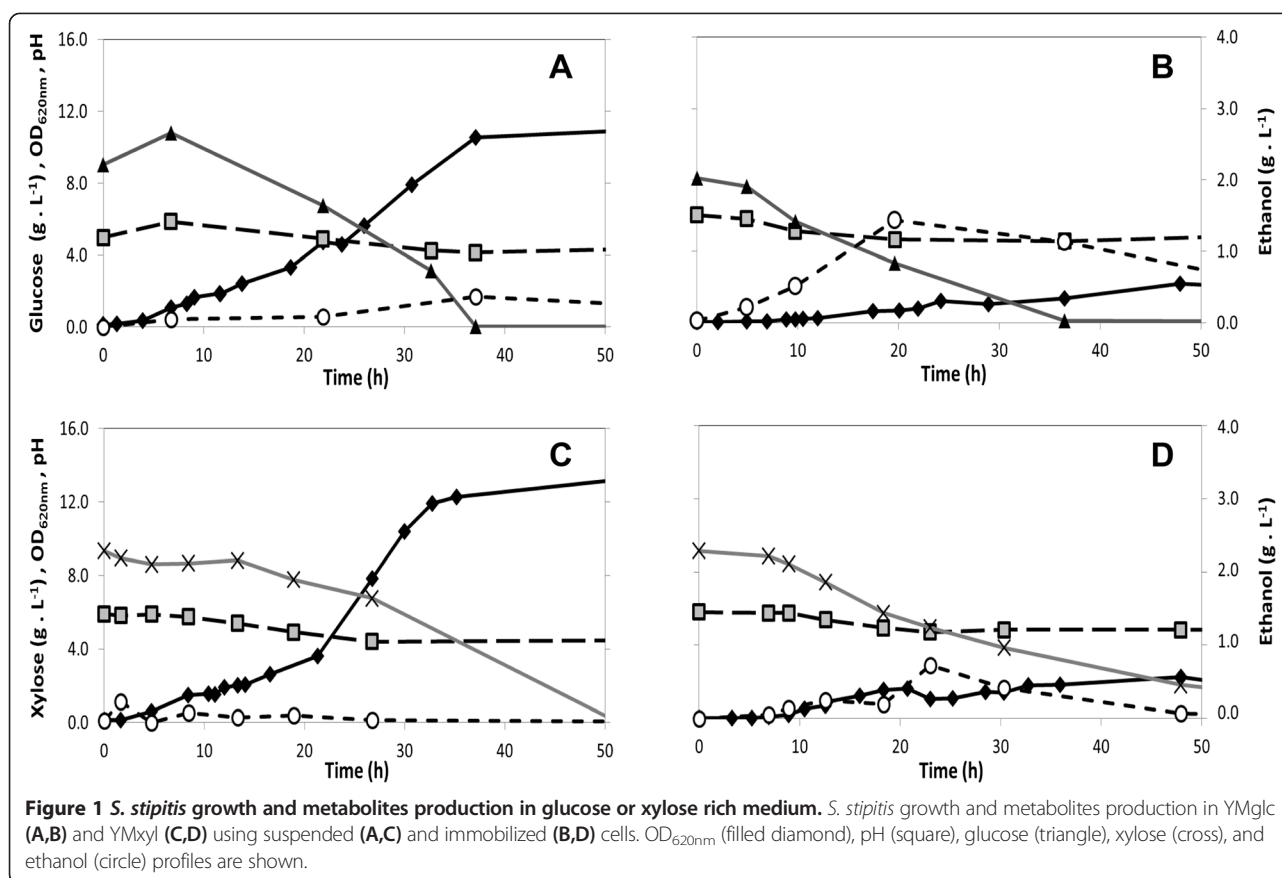
The distribution of cells inside the calcium alginate beads was examined by direct microscopic observation of thin sections. The beads were fixed in 2.5% glutaraldehyde in cacodylate buffer 0.1 M for about 2 h, briefly washed in the same buffer and dehydrated through a graded ethanol series followed by propylene oxide. The beads were then embedded in Spurr's resin TAAB: (TAAB Laboratories Limited, Aldermaston, England) overnight with constant agitation (100 rpm). After polymerization for 8 h at  $70^{\circ}C$ , 1.5- $\mu$ m thick sections were cut with glass knives on an EM UC6 ultramicrotome (Leica Microsystems, Wetzlar, Germany). The sections were stained with toluidine blue and examined by differential interference contrast microscopy on a Zeiss Axioplan 2 imaging light microscope (Zeiss, Jena, Germany) equipped with a DP70 Olympus camera (Shinjuku, Tokyo, Japan).

## Results and discussion

### Cultivations in rich medium

The effect of immobilization of *S. stipitis* in a calcium alginate matrix was first investigated in rich medium using glucose or xylose as sole substrate in shake flasks. The main goals were to analyze whether the immobilization would improve the ethanol production and/or limit substrate uptake and to study the differences in fermentation profile between hexose and pentose sugars under these conditions.

The maximum glucose uptake rate using suspended cells (Figure 1A) was 1.5-fold higher than using immobilized cells (Figure 1B), 0.34 and 0.23 g glucose.L $^{-1}$ .h $^{-1}$ , respectively. This suggested that the calcium alginate matrix limited the mass transfer so that the glucose transfer rate through the immobilizing matrix was slower than the glucose uptake rate of cells inside the beads. Thus, the limiting step was the mass transfer process and not the substrate uptake kinetics. In contrast, in the cultivations using YMxyl (Figure 1C,D) the immobilization did not result in any negative effect on the maximum uptake rate - 0.17 g xylose.L $^{-1}$ .h $^{-1}$  for both suspended and immobilized cells. However *S. stipitis* xylose uptake rates (Figure 1C,D) were lower than those obtained for glucose (Figure 1A,B), since pentose metabolism is slower and less energetic than hexose metabolism [25]. Thus, in this case, the bottleneck was most probably not the substrate available inside the beads



but the lower uptake rate of xylose by *S. stipitis* cells. The limiting step was the pentose uptake kinetics and not the substrate mass transfer. In the cultivations with suspended cells (Figure 1A,C), biomass reached approximately the same level on glucose and xylose and was the main cultivation product, which indicates that most of the carbon was directed towards biomass formation and not towards ethanol production due to the aerobic conditions provided by the shake flask set-up. Increased OD<sub>620nm</sub> was also recorded in the other set-up (Figure 1B,D) despite the immobilization process. This means that some of the beads were disrupted by the stirring or because of cells growth. Alternatively, cells that bound to the outer layer of the matrix during the entrapment process may have been released in the medium by the agitation and aeration levels in the flasks.

When immobilized *S. stipitis* cells were used for the cultivation, there was a significant increase in ethanol production both from glucose and xylose (Figure 1B,D), resulting in higher titer, yield and volumetric productivity. With suspended cells, only 0.43 and 0.18 g ethanol.L<sup>-1</sup> were produced after 50 h in YMglc and YMxyl, respectively, whereas maximum ethanol concentrations of 1.43 g ethanol.L<sup>-1</sup> on glucose and 0.72 g ethanol.L<sup>-1</sup> on xylose were achieved after 20 h with

immobilized cells. After reaching its maximum, ethanol content decreased in both substrates, either because it was used as a carbon source or due to evaporation. This 3.3-fold and 4.0-fold higher titer with immobilized cells using glucose or xylose, respectively, suggests the existence of an O<sub>2</sub> limitation that is likely to arise from the immobilization matrix. The calcium alginate matrix might have limited the dissolved oxygen transfer and thus optimized the oxygen content inside the beads towards microaerophilic conditions that promote fermentative metabolism.

#### Impact of *bdHSSL* inhibitors on *S. stipitis* growth

Pereira et al. in 2012 demonstrated the inability of *S. stipitis* to grow in chemically treated HSSL, due to presence of weak acids such as acetic acid and gallic acid, among other inhibitory compounds. After the biodegradation made by *P. variotii*, *S. stipitis* was able to grow in *bdHSSL* and ethanol production was obtained (0.24 g ethanol.g sugars<sup>-1</sup>). However, there were still some remaining inhibitory compounds present on the *bdHSSL*, affecting the cell viability and performance: lignosulfonates, malic acid, propanoic acid, syringic acid, furoic acids and also acetic acid.

The impact of *bdHSSL* toxicity on *S. stipitis* performance was first evaluated by the determination of maximum

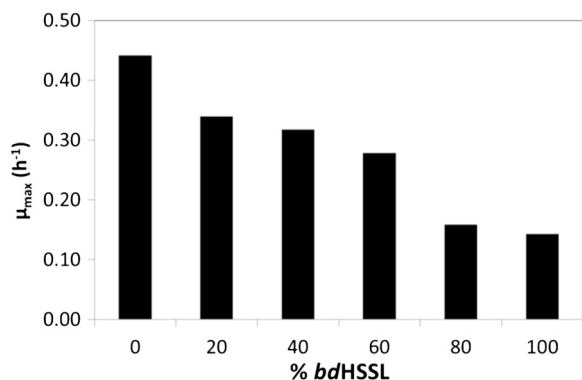


growth rate ( $\mu_{\max}$ ) at six different *bdHSSL* levels (0%, 20%, 40%, 60%, 80%, and 100%, Figure 2). As diluted *bdHSSL* was compensated for sugars and salts in order to avoid nutrient limitation, changes in  $\mu_{\max}$  were assumed to be caused by the presence of the inhibitory compounds only. Without *bdHSSL*, the obtained  $\mu_{\max}$ ,  $0.37 \text{ h}^{-1}$ , was similar and consistent to previously reported values [5]. With 20% *bdHSSL*,  $\mu_{\max}$  decreased to  $0.34 \text{ h}^{-1}$ . Between 20% and 60%,  $\mu_{\max}$  slightly decreased despite the increase in inhibitors levels. Above 60%, the negative impact on growth was significant, with  $\mu_{\max}$  around  $0.15 \text{ h}^{-1}$ . Still *S. stipitis* could grow in 80% and 100% *bdHSSL*. From the screening assays, 60% *bdHSSL* was considered as a good compromise between the final biomass,  $\mu_{\max}$  and the ability to successfully resist to the presence of yeast inhibitors. Hence, 60% *bdHSSL* was used in the following experiments as a pre-adaptation step in order to enhance growth and fermentation efficiencies in *bdHSSL*.

#### Bioreactor cultivations in 100% *bdHSSL* using pre-adapted cells

Batch cultivations in bioreactor were used with 100% *bdHSSL* to evaluate the effects of (i) immobilization in a calcium alginate matrix and (ii) pH control in suspended and immobilized *S. stipitis* cultures. Several metabolites were analyzed by HPLC and the cultivation profiles are shown in Figure 3. Glycerol, furfural and HMF, whose concentrations were below the detection limit, are not discussed.

Using suspended *S. stipitis* (Figure 3A), pH remained stable during the 25 h of cultivation and then it started to slightly increase until 50 h. Glucose and xylose were not completely consumed until the end of the cultivation ( $t_F$ ), remaining approximately 79% of the initial present sugars. The decrease in acetate might indicate that it was not present in a fully inhibitory concentration. The consumption of organic acids was unexpected, since



**Figure 2** Maximum specific growth rate of *S. stipitis* in *bdHSSL* diluted to different percentages. Each % *bdHSSL* was compensated for sugars and salts, being the inhibitory compounds the only difference.

there were monomeric sugars available. Ethanol concentration reached its maximum ( $1.1 \text{ g.L}^{-1}$ ) after 48 h, corresponding to a maximum ethanol yield ( $Y_{E/S}$ ) of  $0.20 \pm 0.00 \text{ g ethanol.g}^{-1}$  substrate and maximum ethanol productivity ( $P_E$ ) of  $27.6 \pm 0.0 \text{ mg ethanol.(L.h)}^{-1}$  (Table 1). Biomass variation suggests that sugars, organic acids and even ethanol were used for growth and cell division. These results were probably arising from the presence of fully aerobic conditions in the bioreactor, since *S. stipitis* favors growth under excess of oxygen and ethanol production is maximized when microaerophilic conditions are present [14,15].

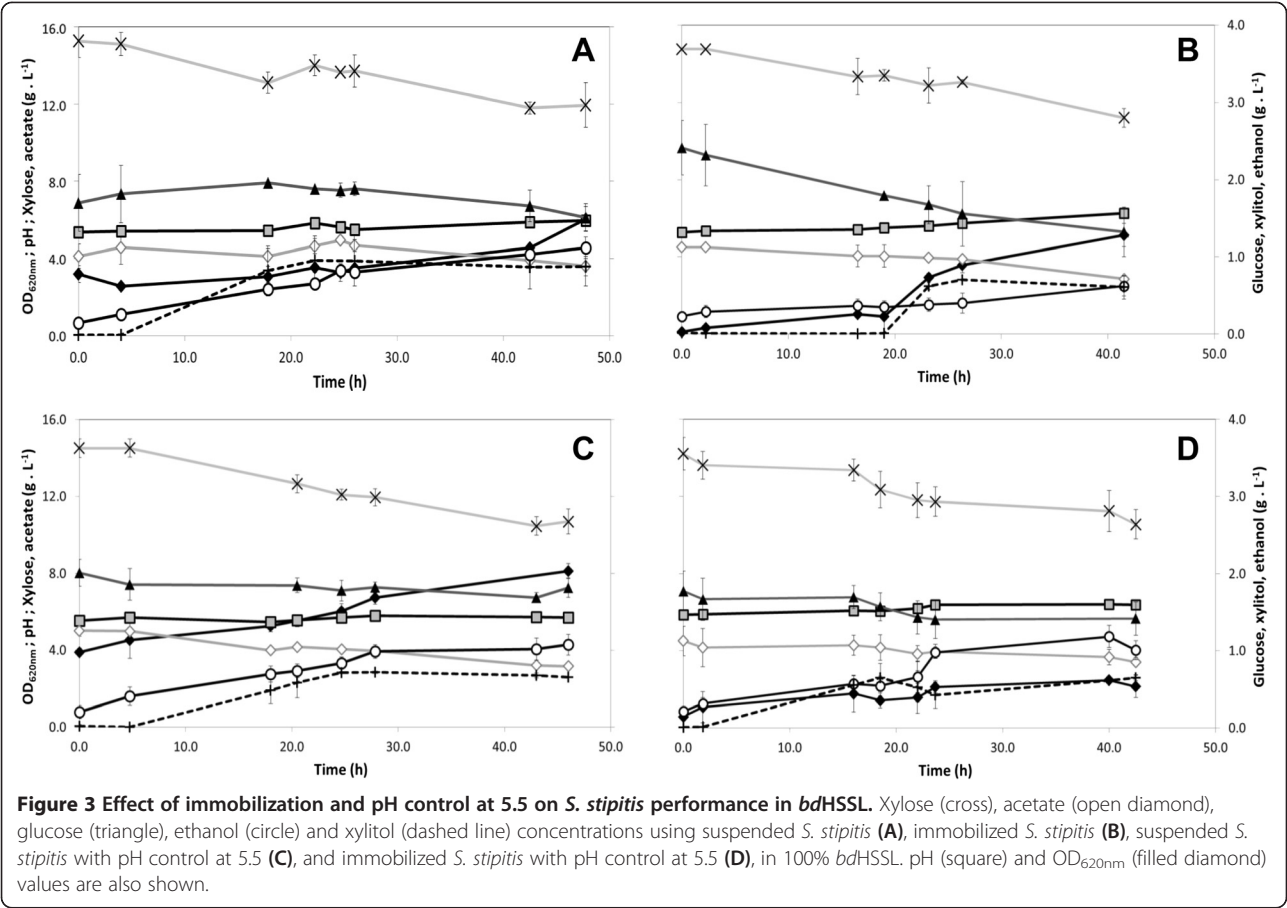
#### Effect of cell immobilization

Figure 3B shows the changes in *S. stipitis* cultivation profile caused by the immobilization. The pH remained stable until the 25 h and then slightly increased from 5.3 to 6.3 at  $t_F$  resulting in a final higher pH than with suspended cells. Unexpectedly, cell immobilization without pH control did not improve ethanol production. In contrast, ethanol production was low and the maximum concentration ( $0.62 \text{ g.L}^{-1}$ ) was achieved after 42 h. The  $Y_{E/S}$  was approximately the same as in suspended culture without pH control but the maximum productivity was reduced 3.6-fold.

During the experiment, the OD significantly increased. During *S. stipitis* entrapment process some cells bound to the outer layer of the matrix. The agitation and aeration levels on the bioreactors caused the release of those cells into the *bdHSSL*. Besides that, some Ca alginate beads were disrupted during the fermentation due to the shear stress on the bioreactors. Before this assay a first trial with immobilized cells in 100% *bdHSSL* was performed without the addition of  $\text{CaSO}_4$ . After 18 h, most of the beads were disrupted and alginate accumulated in the bottom of the bioreactors (data not shown). This result suggested that the anions present in *bdHSSL* bound to the calcium cations that were interacting previously with alginate. Since the amount of lignin sulfonated groups in the SSLs is commonly high, they could act as calcium sequestering agents in the media [26]. Therefore, a previous addition of  $\text{CaSO}_4$  0.2% (m/V) aimed at providing more  $\text{Ca}^{2+}$  in the media, avoiding sequestration of the calcium cations out of the calcium alginate matrix. The results showed that the beads remained stable during more time, but some degradation still occurred probably due to the depletion of  $\text{CaSO}_4$ . Thus, disruption of some calcium alginate barriers resulted in the release of *S. stipitis* cells from beads to the medium that can have created an artificial and continuous supply of fresh cells, these cells being also able to propagate as suspended forms.

#### Effect of pH control

*bdHSSL* is a highly buffered media, meaning that pH should not vary unless significant changes are applied.



Since pH increased after 25 h on assays with both suspended and immobilized cells, cultivations with pH controlled at 5.5 were performed in order to evaluate its effect on *S. stipitis* cultivations. Figure 3C,D presents the time courses for the batch tests performed using suspended and immobilized *S. stipitis*, respectively, in 100% *bdHSSL* with pH control at 5.5. Using suspended cells, the pH maintenance led to a faster consumption of xylose and acetate (Figure 3C). More than 5 g.L<sup>-1</sup> of xylose was consumed in 50 h. Acetate started to be consumed after 20 h and the uptake rate was slightly higher than for suspended culture without pH control (Figure 3A). Maximum ethanol concentration (1.14 g ethanol.L<sup>-1</sup>) was obtained at *t<sub>F</sub>*. More interestingly, at 17 h the

ethanol concentration reached 0.90 g.L<sup>-1</sup>, which is 50% more than the obtained with suspended cells without pH control (0.60 g.L<sup>-1</sup>). This means that with pH control the production of ethanol was more efficient and occurred at a higher rate (*P<sub>E</sub>* was increased by 1.3-fold). The maximum specific ethanol productivity (*P<sub>E/X</sub>*) and the maximum substrate uptake rate (*r<sub>S</sub>*) were also significantly higher with pH control, reinforcing its positive impact on *S. stipitis* performance (Table 1). As in suspended cultures, the positive effect of the pH control in fermentation efficiency was also found in the cultivation with immobilized cells and pH control at 5.5 (Figure 3D). Immobilization together with pH control resulted in the highest *Y<sub>E/S</sub>* in this study,

**Table 1** *S. stipitis* ethanol production and substrates uptake in *bdHSSL* with and without pH control

<i>S. stipitis</i>	pH control	<i>Y<sub>E/S</sub></i>	<i>P<sub>E</sub></i>	<i>P<sub>E/X</sub></i>	<i>r<sub>S</sub></i>
Suspended	No	0.20 ± 0.00	27.6 ± 0.0	0.29 ± 0.03	1.04 ± 0.12
	Yes	0.20 ± 0.01	35.4 ± 1.3	0.39 ± 0.02	1.37 ± 0.39
Immobilized	No	0.19 ± 0.09	7.6 ± 1.4	NA	NA
	Yes	0.26 ± 0.06	27.5 ± 4.9	NA	NA

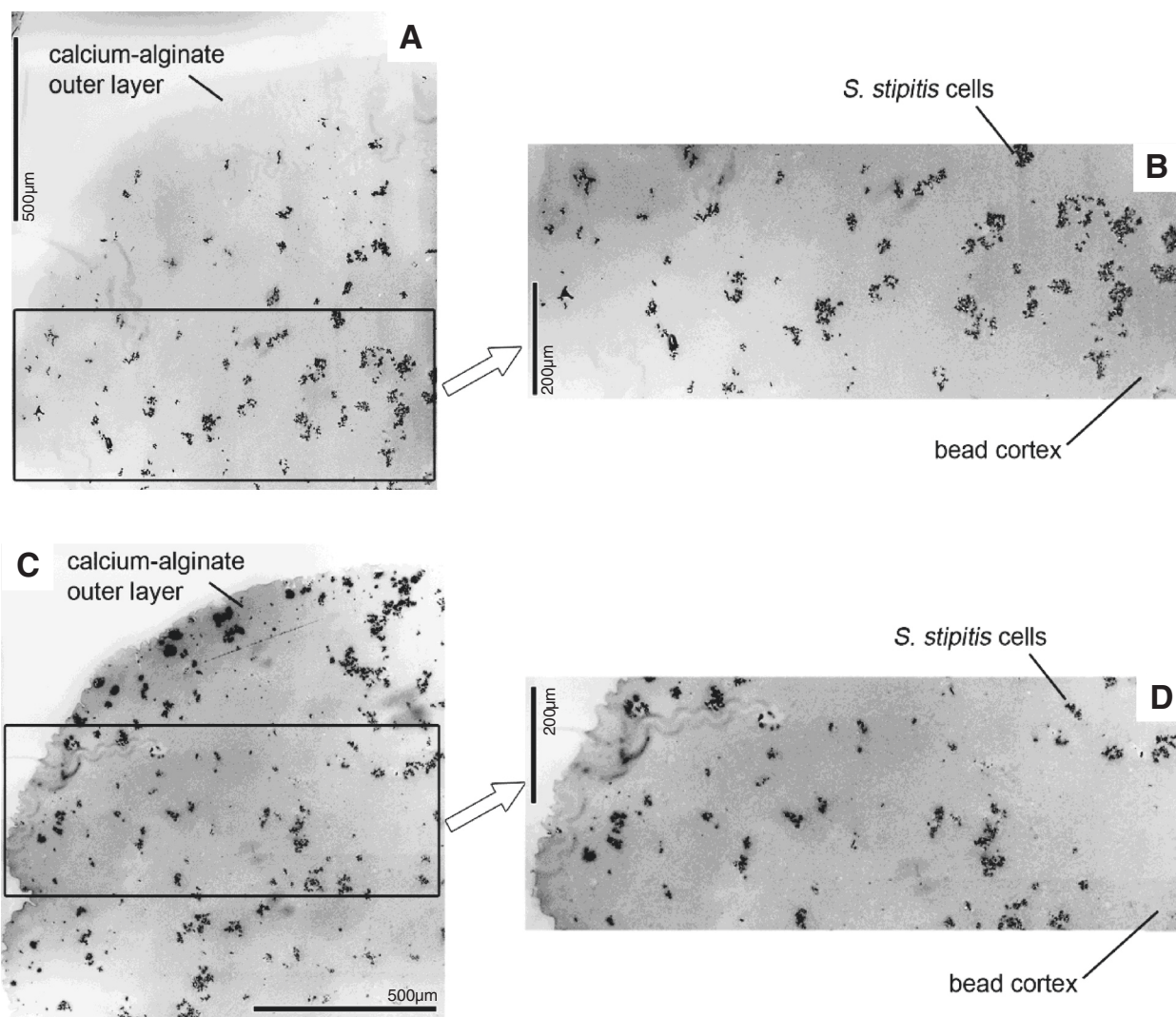
*Y<sub>E/S</sub>*, maximum ethanol yield (g ethanol.(g consumed glucose + xylose)<sup>-1</sup>); *P<sub>E</sub>*, maximum ethanol productivity (mg ethanol.(L.h)<sup>-1</sup>); *P<sub>E/X</sub>*, maximum specific ethanol productivity (mg ethanol.(g CWW.h)<sup>-1</sup>); *r<sub>S</sub>*, maximum substrate uptake rate (mg consumed glucose + xylose.(g CWW.h)<sup>-1</sup>); NA, not applicable.

$0.26 \pm 0.06$  g ethanol.g<sup>-1</sup> substrate. This represents a 1.4-fold improvement on the  $Y_{E/S}$  when compared to the immobilized cultivations without pH control (Table 1).  $P_{E/X}$  and  $r_s$  were not determined for the immobilization assays since the biomass could not be precisely measured due to the residues of the calcium alginate matrix present on the media.

The higher ethanol production suggests that the calcium alginate matrix limited the dissolved oxygen mass transfer and optimized the oxygen content inside the beads, being the *S. stipitis* cells in microaerophilic conditions. This limitation has not occurred in the previous trials, indicating that only under specific and constant pH together with immobilized cells it is possible to reach conditions that favor the fermentative metabolism on the bioreactor. Moreover, the disruption of the beads

during time might have acted as an artificial and continuous supply of fresh cells in the bioreactor. In addition the physical barrier provided by the calcium alginate might have played an important role in terms of tolerance, since the cells were less exposed to the inhibitors (e.g. lignosulfonates) and harsh conditions.

Considering  $OD_{620nm}$  in the immobilization experiments, the biomass increased over time both without and with pH control but in a lower level in the last case. This indicates that pH control at 5.5 and immobilization caused a positive effect on the cell viability in *bdHSSL*. The addition of  $CaSO_4$  to avoid the beads disruption was partially successful since, even with shear stress, there were beads that resisted during all the cultivation. The initial pH at 5.5 contributed also to an improved stability of the calcium alginate matrix, as a higher



**Figure 4** Light micrographs of section through a calcium alginate bead of *S. stipitis* cells. Distribution at the beginning ( $t_0$ ) (A) and at the end of the cultivation ( $t_T$ ) (C). Higher magnification of the marked areas in (A,C) is shown in (B,D), respectively.



number of calcium alginate beads that resisted to the disruption during cultivation with pH control was visible.

#### *S. stipitis* beads examination by light microscopy

Sections of *S. stipitis* beads from  $t_0$  and  $t_F$  were analyzed in light microscopy to evaluate the cells distribution and the effect of *bd*HSSL inside the calcium alginate matrix. In the immobilized cells from the assays with and without pH control no visible changes were detected on the microscope. The results showed on Figure 4 correspond to the fermentations with pH control at 5.5. At  $t_0$  (Figure 4A) the cells were widely spread inside the bead, meaning that the immobilization process occurred as predicted. The shape of the beads was approximately rounded which should facilitate mass transfer. A perfect and uniform round shape and smaller beads size would maximize the efficiency of the transfer and avoid limitations that might occur because of the physical barrier, but this could only be analyzed with more accurate technical equipment [27]. At  $t_F$  there was a higher number of cells close to the calcium alginate outer layer (Figure 4C), indicating that the cells had grown/migrated in the opposite direction of the cortex. There, nutrients and carbon sources are more available and cells in the outer layer have more access to the substrates from the HSSL. However, it was expected that the *S. stipitis* cells would be more active mostly in the cortex area in order to be more protected from the inhibitory compounds. So this result seems to indicate that nutrient limitation has a greater impact than inhibitor stress under the studied conditions. Still the possibility of distinguishing viable cells amongst the total number could not be achieved with the utilized fixation and embedding processes. An improvement in this field or the use of another technique should allow the analysis of the areas where viable yeast cells are located in the end of the fermentation.

#### Conclusions

Cultivations in rich medium, both with glucose or xylose, showed that *S. stipitis* has the potential to be an ethanol producer using fermentative processes. Immobilization in calcium alginate matrix improved the ethanol production by creating aeration levels for *S. stipitis* close to those present in microaerophilic conditions. The screening assays showed differences in *S. stipitis* performance in six different levels of *bd*HSSL and 60% *bd*HSSL enabled a good compromise between the amount of inhibitors and the ability of the yeast to grow on a lignocellulosic-based by-product. Immobilization and pH 5.5, when applied simultaneously, led to a 1.3-fold increase of  $Y_{ES}$  when comparing to the suspended culture without pH control, reaching  $0.26 \text{ g ethanol} \cdot (\text{g glucose} + \text{xylose})^{-1}$ . Without pH control, immobilization seems to have a negative impact

on the ethanol production, probably because it reduces the substrate uptake rate over the matrix. The process to perform the microtome cutting coupled with the microscopic observation allowed the analysis of the cell distribution inside the immobilized beads. To the best of our knowledge, it was the first time that this technique was used for this purpose. During cultivations, the cells have grown and/or migrated in opposite direction of the cortex, suggesting that the lack of nutrients and mass transfer limitations played a more important role than the inhibitors protection. This study contributed to a better knowledge of the influence that immobilization alone or together with pH can have on the metabolites distribution in *S. stipitis* cultivations. Further investigations under controlled microaerophilic conditions should be performed in order to evaluate if the pH control and immobilization have the same positive effects on ethanol production.

#### Abbreviations

*bd*HSSL: bio-detoxified HSSL; CDW: cell dry weight; glc: glucose; HSSLs: hardwood SSLs; SSLs: spent sulfite liquors; xyl: xylose; YM: yeast medium; YNB: yeast nitrogen base.

#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

DPN participated in the design of the study, performed the experimental work and wrote the manuscript. VSN and SRP participated in the design of the project, provided technical and scientific guidance and were involved in the manuscript draft. SCC participated in the design, analysis and interpretation of the *S. stipitis* beads data obtained through light microscopy. AJH helped on the interpretation of the data and figure manipulation. AMRBX conceived the idea, was part of the design of the study and provided scientific guidance and constructive feedback during the draft of the manuscript. All authors read and approved the final manuscript.

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